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During the last year we have concentrated our research in the following areas:

1. The regulation and optimization of the synthesis and production of poly-(β-hydroxyalkanoates) (PHA) in *Rhodospirillum rubrum* and *Pseudomonas oleovorans*. Oxygen concentration affects cell growth, polymer content and cell yield.

2. The biosynthesis and analysis of functionalized PHA from *R. rubrum*, *Alkaligenes eutrophus* and *Pseudomonas oleovorans*. A remarkable functionalized biopolymer β-hydroxy-5-phenylvalerate was produced by *Pseudomonas oleovorans*.

3. A study was initiated on the production and characterization of biodegradable polymers from *Pseudomonas oleovorans*. Keywords: biosynthesis

(See appended research summaries pp. 1-4.)

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Research Summaries for Annual Report Contract N00014-86K-0369
Cellular and Molecular Approaches to Polymer Synthesis of Bacteria.

March 1, 1988 - February 28, 1989

The following personnel have contributed to the project during the last contract year:

Contractors

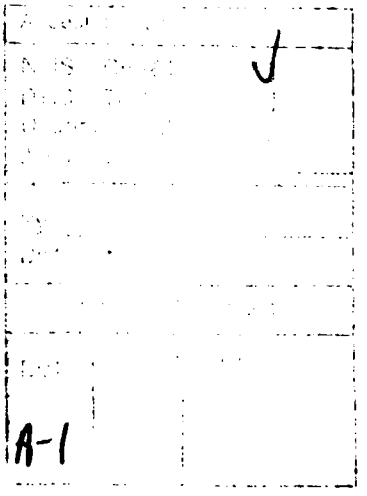
Dr. Robert W. Lenz - Professor of Polymer Science & Engineering
Dr. R. Clinton Fuller - Professor of Biochemistry

Research Associates

Dr. Richard Gross
Dr. Helmut Brandl
Dr. Markus Wolf
Dr. Katharina Fritzsche
Dr. Manuela Benvenuti
Mr. Edward Knee

Students

Mr. Paul Weisbach
Mr. Alex Jesurum
Ms. Young Baek Kim (Korea)
Mr. Herbert Ulmar
Mr. Steven Fagan



Control of PHA synthesis

Recently our research has been focused on the effect of oxygen concentration on PHA production. We are currently using two organisms, *Rhodospirillum rubrum* and *Pseudomonas oleovorans*, to investigate oxygen's role in polymer formation. It has been observed that aeration conditions affect cell growth, polymer content and polymer yield.

Rhodospirillum rubrum was grown anaerobically in the light with acetate as a carbon source. Under these conditions PHA constituted about 2 to 5% of the cellular dry weight. These culture conditions were then altered by the addition of oxygen. When *R. rubrum* is grown in the light with oxygen the photosystem becomes photo-oxidized, a condition stressful to the cell. These changes cause the cell to utilize aerobic metabolic pathways. These experiments showed that after aeration, growth increased as well as polymer production. Cell dry weight increased from 200 to almost 800 mg/l, while PHA yield increased from 20 to 325 mg/l. It was also noted that the pH of the culture increased from 6.8 to about 9.0.

Experiments with *Pseudomonas oleovorans* have shown that, under conditions of limited oxygen, growth is inhibited and polymer production is triggered. In batch cultures, as oxygen concentration approaches zero, cellular PHA content increased from near zero to 20% of the cellular dry weight. A series of experiments has shown at which times, during the growth of the organism, polymer production is greatest and harvesting gives maximum PHA yield. Other experiments have also been conducted which determined optimum nutrient concentration in the growth media. These experiments have strictly defined the growth characteristics of *P. oleovorans*, information which is necessary for establishment of a continuous culture.

These results will be used by others in different areas of the project to increase production of novel PHAs. These experiments also form a foundation upon which future experiments will be based. In particular these results will be used to design continuous culture experiments. Continuous culture techniques will allow us to harvest large volumes of cells, which contain the optimum amount of polymer, and also allow study of biochemical processes at specific points in the growth cycle.

Biosynthesis and Analysis of Functional PHA from *Rhodospirillum* and *Alcaligenes*

The major emphasis is to produce functional biodegradable polymers and new, totally biodegradable polyhydroxyalkanoates, PHAs. The work entails the use of two microorganisms, *Rhodospirillum rubrum* and *Alcaligenes eutrophus*, which metabolize the shorter chain acids (propanoic acid to heptanoic acid).

We investigated the production of polymers with pendent functional groups by *A. eutrophus*, in addition to the well-known copolymer poly(hydroxybutyrate-co-hydroxyvalerate), P(HB-co-HV). Various acids, β-hydroxy acids, and functional acids were used as carbon sources, which were then fed to the bacteria. So far, *A. eutrophus* has produced varying compositions of P(HB-co-HV), and has shown limitations in producing varying PHAs.

R. rubrum shows greater versatility in producing unique PHAs. As with *A. eutrophus*, various carboxylic acids were fed to *R. rubrum*. There was incorporation of longer pendent groups (e.g. propyl groups) and when grown on 4-pentenoic acid, a copolymer of up to 30 mole percent olefin pendent groups was obtained.

Biosynthesis and Analysis of Functional PHA from *Pseudomonas Oleovorans*

Various alkanoates and β-hydroxyalkanoates with an additional functionality, such as methylbranches, doublebonds, ketone, hydroxyl and carboxyl groups, and a derivative with a protected hydroxyl group, were synthesized for use as organic substrates.

These and some commercial alkanoates with phenyl-, p-nitrophenyl-, p-methoxyphenyl-, amino-, bromo- and cyano-substituents were each used as the sole carbon-source to cultivate the microorganism *Pseudomonas oleovorans*, which is known to form PHA (poly-β-hydroxyalkanoates) from straight chain alkanoates like octanoate or nonanoate. Mixtures of octanoate and methyl branched octanoates with varying composition were fed also.

The growth of the bacteria on these substrates was determined by measuring the optical density of the culture and the weight of the lyophilyzed cells. *P. oleovorans* grew on almost all of the substituted alkanoates moderately to well, but sometimes growth started only after an induction period of up to ten days. In some cases the concentration of the carbon source was varied in order to optimize the growth.

The cells were examined for their content of polymer by microscopy, looking for visible inclusions in the cells. The polyester content was determined quantitatively either by GC analysis of the methanolized polymer, after treating the dry cells with sulfuric acid in methanol, or by the weight of the isolated polymer obtained from solvent extraction of the lyophilyzed cells.

Although *P. oleovorans* grew on almost all of the substituted alkanoates, polymer was only formed from 7-methyloctanoate, 5-phenylvalerate, β-hydroxy-6- and β-hydroxy-7-octanoate. As expected polymer was obtained also from the mixtures of octanoate with methyl branched octanoates.

The ^{1}H- and ^{13}CNMR spectra of these polyesters showed that the double bonds, the methyl branches and the phenyl group were incorporated into the side chain of the PHAs. As far as the mixtures of octanoate with methyl octanoates are concerned, branched units were incorporated into the polymer not only in case of the 7-methyloctanoate but also of 6- and 5-methyloctanoate, but no polymer was formed at all if only these substrates were fed without octanoate.

The composition of the polymers and copolymers was determined by GC-analysis of the methanolyzed polyester. To identify the peaks the corresponding methyl-β-hydroxy-alkanoates were synthesized as authentic standards.

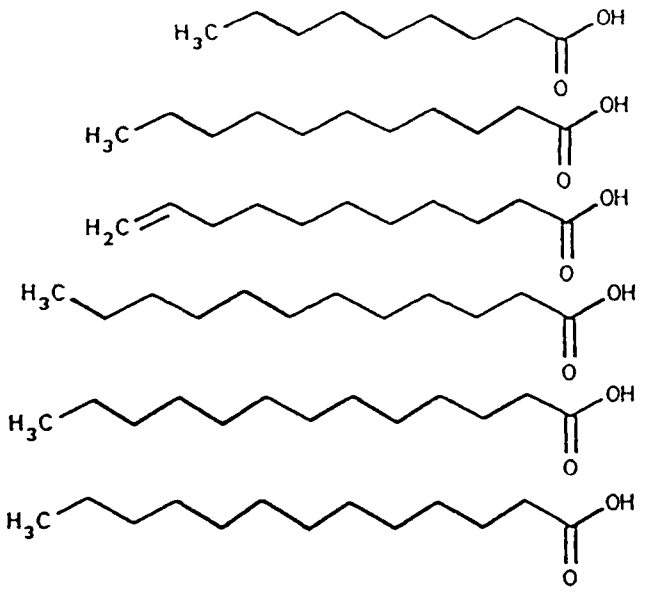
The polymers obtained from the alkanoates and the methyl branched alkanoates contained mainly units of the corresponding β-hydroxyalkanoates, and

to a lesser extent, units which were two carbon-atoms shorter. Furthermore the polyesters obtained from the unsaturated β-hydroxyoctanoates contained also units with a saturated alkyl side chain. The polymer formed from 5-phenylvalerate was poly-β-hydroxy-5-phenylvalerate, a pure homopolymer. This is truly a unique biopolymer and a remarkable result, and this finding offers great potential for a functional polymer of PHA.

The molecular weights of the new polyesters determined by GPC-analysis compared to polystyrene standards ranged from M_W - 200,000 to 300,000 and M_N - 120,000 to 270,000. The polydispersities were very low and varied from 1.5 to 2.0.

The thermal analysis with DSC showed that the polyesters with the branched and the unsaturated side chains have a glass transition at about -40 to -20°C and a low temperature melting transition at approximately 50-60°C. The latter was absent in the second DSC run on cooling down the melt rapidly.

A series of new poly-β-hydroxyalkanoates, PHAs, was also obtained and analyzed using only the compounds listed below as the carbon sources.



The fermentations in this study were carried out on a 12L culture scale, and the reproducibility was checked. Methods to obtain reproducible PHAs from the same carbon source were established. New PHA copolymers were prepared also by using mixtures of two of the compounds above as the carbon source, and homogeneous copolymers containing all of the repeating units from both of the compounds were obtained. The crystalline properties of all of these new PHAs were determined.

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Pseudomonas oleovorans as a Source of Poly(β-Hydroxyalkanoates) for Potential Applications as Biodegradable Polyesters

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Pseudomonas oleovorans **was grown in homogeneous media containing *n*-alkanoic acids, from formate to decanoate, as the sole carbon sources. Formation of intracellular poly(β-hydroxyalkanoates) was observed only for hexanoate and the higher *n*-alkanoic acids. The maximum isolated polymer yields were approximately 30% of the cellular dry weight with growth on either octanoate or nonanoate. In most cases, the major repeating unit in the polymer had the same chain length as the *n*-alkanoic acid used for growth, but units with two carbon atoms less or more than the acid used as a carbon source were also generally present in the polyesters formed. Indeed, copolymers containing as many as six different types of β-hydroxyalkanoate units were formed. The weight average molecular weights of the poly(β-hydroxyalkanoate) copolymers produced by *P. oleovorans* ranged from 90,000 to 370,000. In spite of the higher cell yields obtained with octanoate and nonanoate, the use of hexanoate and heptanoate yielded higher-molecular-weight polymers. These copolyesters represent an entirely new class of biodegradable thermoplastics.**

The ability of bacteria to form intracellular storage granules composed of poly(β-hydroxyalkanoates) (PHA) in general, and poly(β-hydroxybutyrate) (PHB) in particular, has been used as a parameter for the taxonomic classification of the genus *Pseudomonas* (26, 29). Many different types of pseudomonads are able to produce this storage polyester, which is usually formed under nutrient-limiting conditions (8, 27). Recently, it became of industrial interest to evaluate these polyesters as biodegradable thermoplastics for a wide range of possible applications, such as surgical sutures, long-term carriers for drugs, or molded plastics and films (16–18). PHB homopolymer and copolymers of β-hydroxybutyrate and β-hydroxyvalerate are currently being industrially produced by ICI Ltd. for this purpose by the use of *Alcaligenes eutrophus*. These polymers are commercially available under the trade name Biopol (P. A. Holmes, European patent application 0 052 459, Oct. 1981). In addition to *A. eutrophus*, some cyanobacteria (e.g., *Aphanothece* sp.) are also known to form poly(β-hydroxybutyrate-co-β-hydroxyvalerate), but their PHA content is usually very low (5). Physical and mechanical properties of these copolymers, such as stiffness, melting point, extension to break, and resistance to organic solvents, can change considerably as a function of the monomer composition (2, 14, 31).

PHA extracted from sewage sludge and marine sediments showed the presence of β-hydroxyalkanoate units other than β-hydroxybutyrate and β-hydroxyvalerate (13, 25, 31), which suggests the presence of microbial populations in these environments that are capable of producing other types of PHA. However, only *Bacillus megaterium* (13) and *Pseudomonas oleovorans* (9; R. Lageveen, Ph.D. dissertation, University of Groningen, The Netherlands, 1986) are known to incorporate repeating units longer than four or five carbon atoms into their storage polyester.

In the present study, we investigated the ability of *P. oleovorans* to produce various types of PHA homopolymers or copolymers and to incorporate different monomers into its storage polymers. The objectives of this study were: (i) to optimize the growth and culture conditions for PHA production by *P. oleovorans*, including an evaluation of NH_4^+ and a variety of organic substrates for further control of polymer production; (ii) to isolate the various polyesters produced and determine their monomer composition and molecular weights; and (iii) to gain information about the metabolic pathways of the various carbon sources which were used in this study for both growth and PHA production.

MATERIALS AND METHODS

Stock cultures of *P. oleovorans* (ATCC 29347) were maintained on a slightly modified E* medium described previously (Lageveen, dissertation). The medium contained the following (per liter): $(NH_4)_2HPO_4$, 1.1 g; K_2HPO_4, 5.8 g; KH_2PO_4, 3.7 g. Ten milliliters of a 100 mM $MgSO_4$ solution and 1 ml of a microelement solution were added. The microelement solution contained the following (per liter of 1 N HCl): $FeSO_4 \cdot 7H_2O$, 2.78 g; $MnCl \cdot 4H_2O$, 1.98 g; $CoSO_4 \cdot 7H_2O$, 2.81 g; $CaCl_2 \cdot 2H_2O$, 1.67 g; $CuCl_2 \cdot 2H_2O$, 0.17 g; $ZnSO_4 \cdot 7H_2O$, 0.29 g. The carbon source was added to give a final concentration of 10 to 20 mM. The pH was adjusted to 7.0, and the medium was autoclaved. For the stock cultures, 10 mM sodium octanoate was used as the sole carbon source. Solid medium was prepared by addition of 1.5% agar. Cells were grown aerobically in liquid culture for 24 h. A sample was transferred to agar plates and grown for another 24 h. The plates were then stored at 4°C for up to 3 months. Cells from these plates were transferred to liquid medium which was used as an inoculum for all further growth experiments.

Generally, growth experiments were performed under aerobic conditions in 200-ml or 1-liter cultures in a temperature-controlled shaker (New Brunswick Scientific Co., Inc.; 31°C; 150 rpm). After the medium was autoclaved,

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Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential sources for biodegradable polyesters

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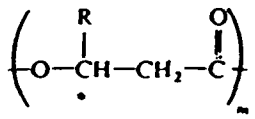
(Received 7 June 1988)

Studies have been carried out in order to optimize growth and culture conditions for the intracellular formation of poly(β-hydroxyalkanoates) (PHA) in the phototrophic, purple, non-sulphur bacterium Rhodospirillum rubrum. *Its potential to produce novel copolymers was investigated. Recently, it has become of industrial interest to evaluate these polyesters as potentially biodegradable plastics for a wide range of possible applications. On an industrial scale, the use of photosynthetic bacteria could harness sunlight as an energy source for the production of these materials.* R. rubrum *was grown anaerobically in the light on different linear and branched β-hydroxycarboxylic acids and various n-alkanoic acids. Under nitrogen-limiting conditions a PHA content of up to 45% of cellular dry weight was detected. When* R. rubrum *was grown on different concentrations of various n-alkanoic acids, intracellular PHA production was detected on all acids used. In most of the cases, the storage polymer contained β-hydroxybutyrate (HB) and β-hydroxyvalerate (HV) monomer units. Grown on n-alkanoic acids with a chain length of four carbon atoms and more,* R. rubrum *produced a copolymer containing the β-hydroxyhexanoate (HC) repeating unit in addition to the HB and HV monomer. Using β-hydroxyheptanoic acid as the carbon source, a polyester which contained HB, HV, HC, and β-hydroxyheptanoate was formed. These copolyesters represent a novel class of biodegradable thermoplastics. The results demonstrate the metabolic flexibility of* R. rubrum *to form many different types of polyesters which might substitute plastics synthesized from petrochemicals.*

Keywords: Biodegradable plastics; poly(β-hydroxyalkanoates); bacterial polymers; *Rhodospirillum rubrum*

Introduction

The phototrophic, purple, non-sulphur bacterium *Rhodospirillum rubrum* is known to produce intracellular energy and carbon storage products which have been generally described as being poly(β-hydroxybutyrate), PHB[1-4]. This particular polymer belongs to the family of poly(β-hydroxyalkanoates), (PHA), (formula 1), which are formed as intracellular inclusions under unbalanced or stressed growth conditions, i.e. in the presence of sufficient carbon or energy source and a limiting nutrient or growth factor[5,6]



(1)

R = n-alkyl pendant group of variable chain length

HB, R = methyl
HV, R = ethyl
HC, R = n-propyl
HH, R = n-butyl
HO, R = n-pentyl
HN, R = n-hexyl
HD, R = n-heptyl
HUD, R = n-octyl
HDD, R = n-nonyl

Thus, reducing equivalents originating from metabolic oxidation processes are stored in a chemically and osmotically inert form[1,7]. The ability of cells to produce PHA is widespread among micro-organisms and a variety of bacterial strains is capable of forming this intracellular polyester[1]. Generally, environmental conditions and physiological abilities of the bacteria

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